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REVIEW PAPER

Role of DREBs in regulation of abiotic stress responses in plants

Charu Lata and Manoj Prasad*

National Institute of Plant Genome Research, Aruna Asaf Ali Marg, New Delhi-110067, India

* To whom correspondence should be addressed. E-mail: manoj_prasad@nipgr.res.in

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Abstract

Abiotic stresses such as drought, high salinity, and cold are common adverse environmental conditions that significantly influence plant growth and productivity worldwide. The phytohormone abscisic acid (ABA) plays an important role in physiological and developmental responses as well as in co-ordinating various stress signal transduction pathways in plants. DREBs (dehydration responsive element binding) are important plant transcription factors (TFs) that regulate the expression of many stress-inducible genes mostly in an ABA-independent manner and play a critical role in improving the abiotic stress tolerance of plants by interacting with a DRE/CRT *cis*-element present in the promoter region of various abiotic stress-responsive genes. This review summarizes recent studies highlighting the role of the DRE-binding family of TFs in the adaptive responses to different abiotic stresses and their structural and functional characters with emphasis on the expression and regulation of DREBs. The practical and application value of DREBs in crop improvement, such as stress tolerance engineering as well as marker-assisted selection (MAS), has also been discussed.

Key words: Abscisic acid, dehydration-responsive element-binding, DRE/CRT, marker-assisted selection, over-expression, transcription factors.

Introduction

As sessile organisms, plants are constantly challenged by a wide range of environmental stresses such as drought, high salt, and temperature change. Growth constraints and stress due to these environmental changes result in reduced productivity and significant crop losses. Drought and salinity together affect more than 10% of arable land, resulting in a more than 50% decline in the average yields of major crops worldwide (Bray *et al.*, 2000). Response to abiotic stresses is a very complex phenomenon as various stages of plant development can be affected by a particular stress and often several stresses simultaneously affects the plant (Chinnusamy *et al.*, 2004). Therefore, the mechanisms underlying stress tolerance and adaptation have long been the focus of intensive research.

Plants usually respond to their changing environment in a complex, integrated way allowing them to respond and adapt to the specific set of conditions and constraints present at a particular time. It involves an array of physiological and biochemical modifications in plants including leaf wilting, reduction in leaf area, leaf abscission, stimulation of root growth, changes in relative water content (RWC), electrolytic leakage (EL), generation of reactive oxygen species (ROS), and accumulation of free radicals which disrupt cellular homeostasis by reacting with lipids, proteins, pigments, and nucleic acids resulting in lipid peroxidation (LP), membrane damage, and the inactivation of enzymes, thus affecting cell viability (Bartels and Sunkar, 2005). Besides this, abscisic acid (ABA), a plant growth regulator and stress hormone, induces leaf stomata closure to reduce water loss through transpiration and decreases the photosynthetic rate in order to improve the water-use efficiency (WUE) of plants. Molecular responses to abiotic stresses, on the other hand, include stress perception, signal transduction to cellular components, gene expression, and, finally, metabolic changes imparting stress tolerance (Agarwal *et al.*, 2006). The genes thus induced by stress not only function in protecting cells

from stress by the production of important metabolic proteins but also in regulating the downstream genes for signal transduction.

Large-scale transcriptome analysis has revealed that these gene products can broadly be classified into two groups (Bohnert *et al.*, 2001; Seki *et al.*, 2002; Fowler and Thomashow, 2002). One group constitutes genes that encode proteins to protect the cells from the effects of water stress. These genes include those that govern the accumulation of compatible solutes (key enzymes for osmolyte biosynthesis like proline, betaine, sugars, etc.); passive transport through membranes and energy-requiring water transport systems (water channel proteins and membrane transporters); and the protection and stabilization of cell structures from desiccation and damage by reactive oxygen species (the detoxification enzymes such as glutathione *S*-transferase, catalase, superoxide dismutase, ascorbate peroxidase, etc.); enzymes for fatty acid metabolism, proteinase inhibitors, ferritin, and lipid-transfer proteins; and other proteins for the protection of macromolecules [LEA (late embryogenesis abundant) protein, osmotin, antifreeze proteins, chaperons, etc.]. It has been suggested that introduction or over-expression of genes encoding LEA proteins, proline synthetase or betaine synthetase, etc. can provide tolerance to drought or high salinity in transgenic plants (Cushman and Bohnert, 2000).

A second group of genes activated by abiotic stresses comprises regulatory proteins that further regulate stress signal transduction and modulate gene expression and, hence, probably function in the stress response. They include various transcription factors (TFs) such as myelocytomatosis oncogene (MYC), myeloblastosis oncogene (MYB), basic leucine zipper (bZIP), NAM, ATAF, and CUC (NAC), dehydration responsive element binding (DREB), etc. suggesting the role of various transcriptional regulatory mechanisms in the stress signal transduction pathways; protein kinases [mitogen activated protein (MAP) kinase, calcium-dependent protein (CDP) kinase, receptor protein kinase, etc.]; protein phosphatases and proteinases (phosphoesterases and phospholipase C, etc.) implicated in the regulation of signal transduction and gene expression (Agarwal *et al.*, 2006; Shinozaki and Yamaguchi-Shinozaki, 2007).

The TFs interact with *cis*-elements in the promoter regions of various stress-related genes to up-regulate the expression of many downstream genes, thus imparting stress tolerance (Agarwal and Jha, 2010). In the *Arabidopsis thaliana* genome only, nearly 1500 TFs are reported which are thought to be involved in stress-responsive gene expression (Riechmann *et al.*, 2000). Microarray analysis data in *Arabidopsis* and in several other plants reveal that there are several pathways that independently respond to abiotic stress (in both an ABA dependent and an ABA-independent manner), thus forming a highly complex gene network (Fowler and Thomashow, 2002; Umezawa *et al.*, 2006).

Role of ABA in stress-responsive gene expression

ABA is an important plant hormone that plays a regulatory role in many physiological processes in plants, such as embryo maturation, seed development, seed and bud dormancy, seed germination, root growth, fruit ripening, regulation of stomatal aperture, and the activation of stress-responsive genes (Agarwal and Jha, 2010). Increased levels of ABA are triggered by a variety of environmental stresses such as drought, water stress, salinity, cold, desiccation, heat stress, and wounding. Further, it is also proved that ABA is a major physiological signal that induces drought and high salinity responses (Gomez *et al.*, 1988; Verslues and Bray, 2006; Farooq *et al.*, 2009; Cutler *et al.*, 2010; Hubbard *et al.*, 2010). The action of ABA, therefore, not only involves the regulation of developmental pathways but also controls many stress-adaptation responses such as the activation of genes responsible for osmotic adjustment, ion compartmentalization, root hydraulic conductivity, the regulation of shoot and root growth, limiting transpiration rate and wilting, thus reducing water loss in the plants (Verslues and Zhu, 2005; Pospíšilová *et al.*, 2009). It is also involved in the modification of gene expression, and a number of stress-responsive genes are up-regulated by ABA during osmotic imbalance (Ingram and Bartels, 1996).

Although several genes are induced in response to dehydration and cold stress on exogenous ABA treatment (Zhu, 2002; Shinozaki *et al.*, 2003), there are also many genes that do not respond to such treatments (Zhu, 2002; Yamaguchi-Shinozaki and Shinozaki, 2005) suggesting the existence of both ABA-dependent and -independent signal transduction cascades. DRE/CRT is one of the major *cis*-acting elements which function in ABA-responsive or non-responsive gene expression during abiotic stresses (Nakashima and Yamaguchi-Shinozaki, 2010).

ABA-dependent signalling systems have been described as pathways that mediate adaptation to stress by the activation of at least two different regulons (a cluster of genes controlled by a certain type of TF can be identified: (i) the AREB/ABF (ABA-responsive element-binding protein/ABA-binding factor) regulon; and (ii) the MYC/MYB regulon (Abe *et al.*, 1997; Busk and Pagés, 1998). On the other hand, ABA-independent regulons are: (i) the CBF/DREB (cold-binding factor/dehydration responsive element binding) regulon; and (ii) the NAC and ZF-HD (zinc-finger homeodomain) regulon (Saibo *et al.*, 2009). In addition, previous studies have identified the existence of both ABA-dependent and -independent pathways of stress response and function through members of the AP2/EREBP (ERF) family of TFs (Yamaguchi-Shinozaki and Shinozaki, 1994; Kizis and Pagés, 2002). Although these different stress response pathways usually function independently, it is possible that some level of cross-talk certainly exists between them (Fig. 1).

Other than the above-mentioned regulons, some other TFs such as WRKY, HARDY, Zinc fingers etc. are also involved in abiotic stress tolerance responses and key regulatory networks in plants (for details see Lata *et al.*, 2011*b*). However, until now, the best studied group of TFs in abiotic stresses is the DREB genes since it activates the expression of many target genes that are responsible for controlling correlated characters such as osmoprotection

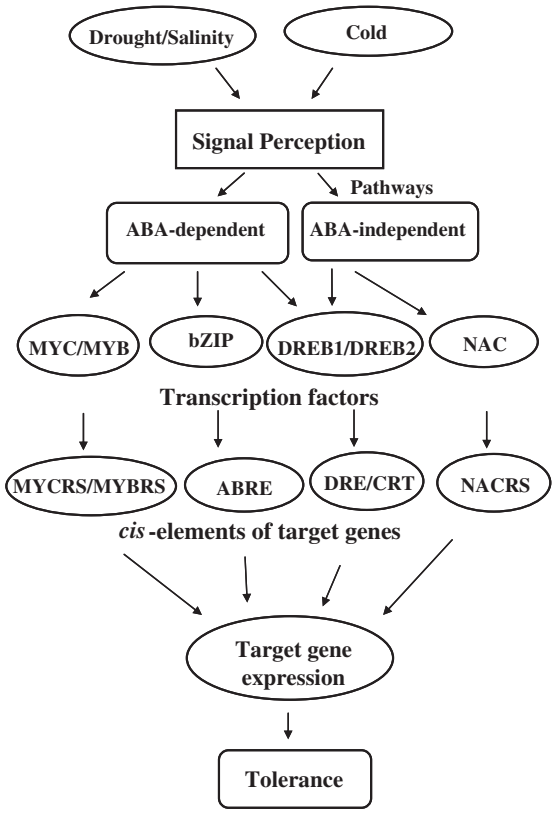

Fig. 1. A schematic representation of stress signal perception and gene expression via ABA-dependent and independent pathways at cellular level in plants (based on well-known concepts).

and metabolism (Hussain *et al.*, 2011). Thus, this review specifically focuses on the DREB proteins and their role in regulating the abiotic stress responses in plants with an emphasis on *Arabidopsis*, grasses, and other crop plants as well as their utility in crop improvement programmes through genetic engineering and marker-assisted selection (MAS).

The dehydration responsive elements

Extensive molecular analyses have revealed the presence of specific *cis*-elements that mediate the activation of various genes under different environmental stresses. The dehydration responsive element (DRE) with a 9 bp conserved core sequence (5'-TACCGACAT-3') was first identified in the promoter of the drought-responsive gene *rd29A* (Yamaguchi-Shinozaki and Shinozaki, 1993). Protein factors from nuclear extracts of salt-stressed and unstressed *Arabidopsis* plants were designated as DRE binding factor-1 or DRBF-1 as they specifically bind to DRE sequence in gel-shift assays thus, resulting in the discovery of the DREB transcription factors (Yamaguchi-Shinozaki and Shinozaki, 1994). Since then DREs have been reported to be involved in various abiotic stress responses through both ABA-dependent and ABA-independent pathways (Yamaguchi-Shinozaki and Shinozaki, 1994; Busk *et al.*, 1997; Liu *et al.*, 1998; Kizis and Pagès, 2002 Dubouzet *et al.*, 2003). This element is essential for the induction of *rd29A* gene expression by osmotic stresses such as drought and high salinity as well as by low temperature, but not ABA (Yamaguchi-Shinozaki and Shinozaki, 1994 Saleh *et al.*, 2005).

Subsequently, the C-repeat responsive element (CRT) with the core sequence 5′-CCGAC-3′ was identified in the promoter of cold-inducible genes from *Arabidopsis* (Saleh *et al.*, 2005). This sequence is related to the DRE motif and is essential for the induction of several genes by low temperature. The CRT motif and the low temperature-responsive element (LTRE) were identified in the promoters of cold-regulated genes from *Arabidopsis* such as *kin1, kin2*, and *rab18,* and were also responsible for the regulation of *BN115* and *WCS120* from *Brassica napus* and wheat, respectively (Kurkela and Borg-Franck, 1992; Lang and Palva, 1992; Baker *et al.*, 1994; Jiang *et al.*, 1996; Ouellet *et al.*, 1998). These are activated in an ABA-independent manner during drought and cold stress in ABA-deficient and ABA-insensitive mutants of *Arabidopsis*. However, it has also been suggested that some DRE/CRT motifs can respond to an ABA-dependent pathway (Haake *et al.*, 2002). The DRE2 of the maize *rab17* promoter, for example, is involved in ABA-dependent responses to osmotic stress with a typical core motif of 5′-ACCGAC-3′ and was identified in the embryos and leaves (Busk *et al.*, 1997). Additionally, the DRE1 *cis*-element (5′-ACCGAG-3′) has also been identified in the *rab17* promoter mediating an ABA-dependent regulation in the embryo, but not in vegetative tissues in response to drought stress (Busk *et al.*, 1997; Busk and Pagès, 1998 Saleh *et al.*, 2005).

Identification, expression, and structural analysis of the DREB transcription factors

The DREB proteins namely, DREB1 and DREB2, involved in two separate signal transduction pathways under low temperature and dehydration, respectively, are important APETALA2 (AP2)/ethylene responsive factor (ERF) plant TFs that induce a set of abiotic stress-related genes. The large AP2/ERF family of TFs in *Arabidopsis* was characterized on the basis of the number of repetitions and the sequence of the AP2 domain (Sakuma *et al.*, 2002). The 145 members of this family in *Arabidopsis* were classified into five subfamilies namely, DREB, ERF, AP2, RAV, and others (Table 1). The AP2/ERF superfamily of rice, however, can be divided into three families based on sequence similarity and numbers of domains: AP2, ERF (the ERF subfamily and the CBF/DREB subfamily), and RAV (Nakano *et al.*, 2006).

DREB genes play an important role in the ABA-independent stress-tolerance pathways that induce the expression of various stress-responsive genes in plants. The first isolated cDNAs encoding DRE binding proteins, CBF1 (CRT binding factor1), DREB1A and DREB2A were first isolated by using yeast one-hybrid screening (Stockinger *et al.*, 1997; Liu *et al.*, 1998) from *Arabidopsis*. Since then, numerous DREB genes have been isolated from a number of plants (Table 2). These proteins specifically bind to the DRE sequence and activate the expression of genes driven by it.

DREB1B/*CBF1*, *DREB1A*/*CBF3*, and *DREB1C*/*CBF2* genes lie in tandem on chromosome 4 of *Arabidopsis* (Gilmour *et al.*,

Table 1. Classification of AP2/ERF family of transcription factors (based on Sakuma *et al.*, 2002).

AP2/ERF subfamilies	No. of AP2/ ERF domain	Subgroup	Additional domain/motif	No. of genes
AP2	2		-	14
	1			3
DREB	1	A-1 to A-6	Conserved WLG motif	56
ERF	1	B-1 to B-6	Conserved WLG motif	65
RAV	1		B3 domain	6
Others (AL079349)	1		Lack WLG motif	1
Total				145

1998; Liu *et al.*, 1998). *Arabidopsis* also contains two DREB2 proteins namely, DREB2A and DREB2B (Liu *et al.*, 1998). DREB1/DREB2-homologous genes have also been isolated from several grasses such as rice, wheat, barley, maize, sorghum, rye, oat, and perennial ryegrass (Nakashima *et al.*, 2009). In important cereal crops like wheat and barley, a number of *CBF* homologues have been mapped to the Fr-2 chromosomal region (Skinner *et al.*, 2005; Miller *et al.*, 2006). A functional Fr-A1 allele plays an important role in regulating the CBF-mediated *Cor*/*Lea* gene expression in wheat (Kobayashi *et al.*, 2005). Shukla *et al.* (2006) reported the isolation and characterization of a gene (*CAP2*) from chickpea (*Cicer arietinum*) encoding a novel AP2-family TF which is relatively small in comparison to most of the well-studied DREB family members. Detailed functional study on small AP2 proteins such as CAP2 and soybean GmDREB may open up new areas in the plant developmental process. Recently, a novel DREB2-like gene, *SiDREB2*, associated with dehydration stress tolerance, has been isolated from foxtail millet (*Setaria italica*) (Lata *et al.*, 2011*a*).

Expression of the *Arabidopsis DREB1*/*CBF* genes is induced by cold, while the *DREB2* genes are induced by dehydration, high-salinity, and heat stresses generally (Fig. 2) (Liu *et al.*, 1998; Shinwari *et al.*, 1998; Nakashima *et al.*, 2000). However, *CBF4*/*DREB1D*, *DREB1E*/ *DDF2*, and *DREB1F*/*DDF1* are induced by osmotic stress, suggesting the existence of cross-talk between the DREB1 and the DREB2 pathways (Haake *et al.*, 2002; Nakashima *et al.*, 2009).

According to the structure characteristic of DREB TFs, the subfamily of DREB TFs can be further divided into six subgroups from A-1 to A-6 (Sakuma *et al.*, 2002). The DREB TFs contain a highly conserved AP2/ERF DNA-binding domain across the plant kingdom including *Arabidopsis*, rice, soybean, chickpea, tomato, tobacco, and millets (Lata *et al.*, 2011*a*). The three-dimensional structure of the *Arabidopsis* AtERF1 AP2 domain (PDB ID: 1GCC) was solved by a heteronuclear multi-dimensional nuclear magnetic resonance (NMR) technique (Allen *et al.*, 1998). The domain consists of a three-stranded β-sheet and one α-helix running almost parallel to the β-sheet, and it contacts DNA via Arg and Trp residues located in the β-sheet (Magnani *et al.*, 2004). The structure and characteristics largely hold true for AP2 domain-containing proteins from other crops as well. A recent study showed AP2-DNA binding domain of *SiDREB2* from foxtail millet sharing the same conserved features (Lata *et al.*, 2011*a*). Two conserved functional amino acids (valine and glutamic acid) at the 14th and 19th residues, respectively, are present in the DNA binding domain and are thought to be crucial sites for the binding of DREBs to the DRE core sequences (Liu *et al.*, 1998). An alkaline N-terminal amino acid region, which acts as a nuclear localization signal (NLS), and a conserved Ser/Thr-rich region adjacent to the AP2/ERF DNA binding domain are also mostly present. Ser/Thr-rich region is considered to be responsible for phosphorylation of DREB proteins (Liu *et al.*, 1998; Agarwal *et al.*, 2006). The proteins also contain an acidic C-terminal region which is predicted to be functional in *trans*-activation activity (Stockinger *et al.*, 1997). Most of the positively charged residues are conserved at the N-terminal domain. The NLS 'PKRPAGRTK-FRETRHP', a DSAW motif immediately flanking the ERF/AP2 domain and a conserved LWSY motif at the end of the C-terminal are present in most of the DREB1-type proteins (Cong *et al.*, 2008). Similarly, A-2 and A-3 subgroup proteins possess a PKK-like NLS sequence RKXPAKGSKKGCM-XGKGGPENXXA and RKXXXXKGGPXNXKF almost up to the AP2/ERF domain, however, unlike A-1 subgroup members they do not have a conserved motif close to the C-terminal of the AP2-domain (Zhou *et al.*, 2010). A systematic phylogenetic analysis has been carried out, based on the similarities of AP2 domains in the DREB subfamily proteins isolated from various plant species, using MEGA 4.0 by the Neighbor–Joining method (Fig. 3). The phylogenetic analysis showed that all the six groups within the DREB subfamily can easily be classified on the basis of AP2 domains as suggested by Sakuma *et al.* (2002). It was also observed that AP2 domains can easily dichotomize monocots from dicots. This functional conservation makes them important targets for crop improvement for abiotic stress tolerance through genetic engineering and plant breeding.

Role of DREB1/CBFs in cold-responsive gene expression

The *Arabidopsis* DREB1 subgroup consists of six genes (Sakuma *et al.*, 2002). *DREB1A*/*CBF3*, *DREB1B*/*CBF1*, and *DREB1C*/*CBF2* are strongly and transiently induced by low temperature stresses (Gilmour *et al.*, 1998; Fowler and Thomashow, 2002). Interestingly, it was observed that the activation of *CBF1–CBF3* genes in response to low temperature is gated by the circadian clock, suggesting their regulation has aspects in common with the regulation of *Arabidopsis* chlorophyll *a*/*b*-binding (*CAB*) genes (Fowler *et al.*, 2005). *OsDREB1A*, *OsDREB1B*, *OsDREB1C*, and *OsDREB1D*, respectively, have been isolated from rice (Dubouzet *et al.*, 2003). A DREB1/CBF-type TF, *ZmDREB1A* was also identified in maize (Qin *et al.*, 2004).

Competitive DNA binding assays revealed that AtDREB1A protein could bind to both ACCGAC and GCCGAC with the

Table 2. Response of DREB genes to various stresses.

DREB TFs	Species	Accession no.	Stress response	References
DREB1A	*Arabidopsis thaliana*	AB007787	Cold	Liu *et al.*, 1998
DREB2A	*Arabidopsis thaliana*	AB007790	Drought, Salt, ABA	Liu *et al.*, 1998
DREB2C	*Arabidopsis thaliana*	At2g40340	Salt, Mannitol, Cold	Lee *et al.*, 2010
CBF1	*Arabidopsis thaliana*	U77378	Cold	Gilmour *et al.*, 1998
CBF2	*Arabidopsis thaliana*	AF074601	Cold	Gilmour *et al.*, 1998
CBF3	*Arabidopsis thaliana*	AF074602	Cold	Gilmour *et al.*, 1998
CBF4	*Arabidopsis thaliana*	AB015478	Drought, ABA	Haake *et al.*, 2002
OsDREB1A	*Oryza sativa*	AF300970	Cold, Salt, Wounding	Dubouzet *et al.*, 2003
OsDREB1B	*Oryza sativa*	AF300972	Cold	Dubouzet *et al.*, 2003
OsDREB1C	*Oryza sativa*	AP001168	Drought, Salt, Cold, ABA, Wound	Dubouzet *et al.*, 2003
OsDREB1D	*Oryza sativa*	AB023482	None	Dubouzet *et al.*, 2003
OsDREB2A	*Oryza sativa*	AF300971	Drought, Salt, faintly to Cold, ABA	Dubouzet *et al.*, 2003
OsDREB1F	*Oryza sativa*		Drought, Salt, Cold, ABA	Wang *et al.*, 2008
OsDREB2B	*Oryza sativa*		Heat, Cold	Matsukura *et al.*,2010
OsDREB2C	*Oryza sativa*	AK108143	None	Matsukura *et al.*, 2010
OsDREB2E	*Oryza sativa*		None	Matsukura *et al.*, 2010
OsDREBL	*Oryza sativa*	AF494422	Cold	Chen *et al.*, 2003
TaDREB1	*Triticum aestivum*	AAL01124	Cold, Dehydration, ABA	Shen *et al.*, 2003
WCBF2	*Triticum aestivum*		Cold, Drought	Kume *et al.*, 2005
WDREB2	*Triticum aestivum*	BAD97369	Drought, Salt, Cold, ABA	Egawa *et al.*, 2006
HvDRF1	*Hordeum vulgare*	AY223807	Drought, Salt, ABA	Xue and Loveridge, 2004
HvDREB1	*Hordeum vulgare*	DQ012941	Drought, Salt, Cold	Xu *et al.*, 2009
ZmDREB2A	*Zea mays*	AB218832	Drought, Salt, Cold, Heat	Qin *et al.*, 2007
PgDREB2A	*Pennisetum glaucum*	AAV90624	Drought, Salt, Cold	Agarwal *et al.*, I., 2007
SbDREB2	*Sorghum bicolor*	ACA79910	Drought	Bihani *et al.*, 2011
SiDREB2	*Setaria italica*	HQ132744	Drought, Salt	Lata *et al.*, 2011a
CaDREB-LP1	*Capsicum annum*	AY496155	Drought, Salt, Wounding	Hong and Kim, 2005
AhDREB1	*Artiplex hortensis*		Salt	Shen *et al.*, 2003b
GmDREBa	*Glycine max*	AY542886	Cold, Drought, Salt	Li *et al.*, 2005
GmDREBb	*Glycine max*	AY296651	Cold, Drought, Salt	Li *et al.*, 2005
GmDREBc	*Glycine max*	AY244760	Drought, Salt, ABA	Li *et al.*, 2005
GmDREB	*Glycine max*	AF514908	Drought, Salt	Shiqing *et al.*, 2005
GmDREB2	*Glycine max*	ABB36645	Drought, Salt	Chen *et al.*, 2007
PpDBF1	*Physcomitrella patens*	ABA43697	Drought, Salt, Cold, ABA	Liu *et al.*, 2007
PNDREB1	*Arachis hypogea*	FM955398	Drought, Cold	Mei *et al.*, 2009
CAP2	*Cicer arietinum*	DQ321719	Drought, Salt, ABA, Auxin	Shukla *et al.*, 2006
DvDREB2A	*Dendrathema*	EF633987	Drought, Heat, ABA, Cold	Liu *et al.*, 2008
DmDREBa	*Dendronthema×moriforlium*	EF490996	Cold, ABA	Yang *et al.*, 2009
DmDREBb	*Dendronthema×moriforlium*	EF487535	Cold, ABA	Yang *et al.*, 2009
PeDREB2	*Populus euphratica*	EF137176	Drought, Salt, Cold	Chen *et al.*, 2009
SbDREB2A	*Salicornia brachiata*	GU592205	Drought, Salt, Heat	Gupta *et al.*, 2010

same efficiency; however, OsDREB1A protein showed preferential binding to GCCGAC compared with ACCGAC (Sakuma *et al.*, 2002; Dubouzet *et al.*, 2003). However, although the *Aloe DREB1* can bind to the DRE *cis* element it may also bind to other *cis*-elements effectively and, hence, can function in a new cold-induced signal transduction pathway (Wang and He, 2007). A similar result was also observed for *OsDREBL* which did not bind effectively to the CRT/DRE motif (Chen *et al.*, 2003).

Temporal gene expression studies of DREB/CBF genes in various crops have revealed that these are induced by abiotic stresses particularly low temperature, however, at different time periods (Table 2). For example, *AtDREB1* expressed within 10 min at 4 °C (Liu *et al.*, 1998). The transcripts of *CBF* genes were detectable after 30 min of exposure to 4 °C with maximum expression at 1 h (Medina *et al.*, 1999). *CBF1*/*DREB1B* and *CBF3*/*DREB1A* transcripts accumulated after 15 min of cold treatment while *CBF2*/*DREB1C* transcripts accumulated at a slower rate with maximum expression after 2.5 h of cold exposure and then gradually declined (Novillo *et al.*, 2004). However, the *CBF4* TF in *Arabidopsis* was rapidly induced during drought and ABA treatment but not by cold stress (Haake *et al.*, 2002). *OsDREB1A* and *OsDREB1B* were induced soon after cold exposure (within 40 min) but do not respond to ABA treatment. *OsDREB1A* was induced within 5 h of salt stress whereas *OsDREB1C* was constitutively expressed during stress. *OsDREB1D* expression was not detected with or without any stress (Dubouzet *et al.*, 2003; Agarwal *et al.*, 2006). *OsDREBL* also accumulated quickly within 30 min

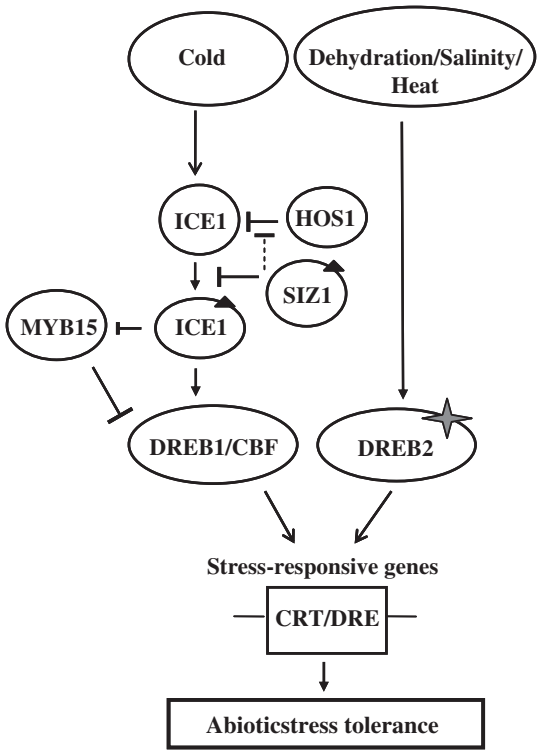

Fig. 2. A typical representation of the induction of abiotic-stress-inducible genes with the CRT/DRE *cis*-element in their promoters (based on well-known concepts from *Arabidopsis*). Transcription factor modifying enzymes are represented as small triangles. The star corresponds to post-translational modification of DREB2.

in response to low temperature, but not in response to ABA, NaCl, and dehydration (Chen *et al.*, 2003). The expression of the *WCBF2* gene from wheat was induced rapidly by low temperature and drought but not by ABA (Kume *et al.*, 2005). In *Arachis hypogaea*, *PNDREB1* was strongly up-regulated by treatments with low temperature, and also responded to dehydration (Mei *et al.*, 2009). However, *Ca-DREBLP1* from hot pepper was rapidly induced by dehydration and high salinity but was not at all affected by cold stress (Hong and Kim, 2005).The expression of *PpDBF1* was also induced by NaCl, cold, drought, and ABA treatments in *Physcomitrella patens* (Liu *et al.*, 2007).

The induction of *DREB*/*CBF* transcripts is organ-specific and proportional to the length of the stress treatment. *AhDREB1* was highly expressed in roots but less in stems and leaves in response to salt stress (Shen *et al.*, 2003*b*). *OsDREB1F* was constitutively expressed in almost all the tissues and organs, including young leaves, young roots, mature leaves, mature roots, young panicles, and callus with higher expression in panicles and callus than in the other tissues (Wang *et al.*, 2008). Expression of the *HvDREB1* gene in barley leaves was significantly induced by salt, drought, and low-temperature (Xu *et al.*, 2009). *GmDREBa* and *GmDREBb* were also induced by cold, drought, and salt in leaves of soybean seedlings while expression of *GmDREBc* was high in roots following drought, salt, and ABA treatments (Li *et al.*, 2005).

Role of DREB2 proteins in drought, salinity, and heat-responsive gene expression

Among the DRE-binding proteins, the DREB2 subfamily is induced by drought and high-salinity stress indicating their important role in stress-responsive gene expression (Table 2). The *DREB2A* and *DREB2B* were first isolated as cDNAs encoding DRE/CRT-binding protein from *Arabidopsis* (Liu *et al.*, 1998). However, among the eight DREB2-type proteins, *DREB2A* and *DREB2B* were thought to be major transcription factors that function under osmotic stresses (Nakashima *et al.*, 2000; Sakuma *et al.*, 2002). Though *DREB1* genes are widely investigated in many crops in response to different abiotic stresses, the studies did not proceed as rapidly for *DREB2* expression. *DREB2* homologous genes have been isolated from economically important cereal crops also such as rice, wheat, barley, maize, pearl millet, and foxtail millet (Dubouzet *et al.*, 2003; Xue and Loveridge, 2004; Egawa *et al.*, 2006; Agarwal *et al.*, 2007; Qin *et al.*, 2007; Lata *et al.*, 2011*a*). *DREB2* transcripts were found to be regulated by alternative splicing in barley, wheat, maize, and rice with most of them showing transactivation abilities in yeast or plant cells (Dubouzet *et al.*, 2003; Egawa *et al.*, 2006; Agarwal *et al.*, 2007; Qin *et al.*, 2007). *PgDREB2* from pearl millet was shown to be phosphorylated by total cell extracts and could not bind to DRE/CRT sequence (Agarwal *et al.*, 2007).

The expression of *Arabidopsis DREB2A* and its homologue *DREB2B* were induced by dehydration and high salt stress, but not by cold stress and exogenous ABA (Liu *et al.*, 1998; Nakashima *et al.*, 2000). ABA, mannitol, and cold treatments had little effect on *DREB2C* expression but an elevated level of DREB2C mRNA was detected after 250 mM salt treatment (Lee *et al.*, 2010). *OsDREB2A* transcript was induced within 24 h of dehydration and 250 mM salt stress but weakly responded to ABA and cold stress (Dubouzet *et al.*, 2003). A comprehensive analysis of all five *OsDREB2*s from rice revealed that *OsDREB2A* accumulated to the highest levels under the non-stress condition, and its expression was increased slightly by high temperature, drought, and high salinity treatments, but not by low temperature (Matsukura *et al.*, 2010). The *OsDREB2B* transcript level was markedly increased especially after 20 min of high temperature and 24 h of low temperature stress, respectively. The transcript levels of *OsDREB2C* and *OsDREB2E* were low under control conditions and were not strongly induced by the abiotic stresses (Matsukura *et al.*, 2010). Wheat *TaDREB1* and *WDREB2*, maize *ZmDREB2A*, and pearl millet *PgDREB2* are responsive to cold stress as well, whereas foxtail millet *SiDREB2* was not (Dubouzet *et al.*, 2003; Shen *et al.*, 2003*a*; Egawa *et al.*, 2006; Agarwal *et al.*, 2007; Qin *et al.*, 2007; Lata *et al.*, 2011*a*). *ZmDREB2A* also responded to high temperature (Qin *et al.*, 2007). Sorghum SbDREB2 showed induction at 1 h exposure to drought, after which expression gradually dropped to basal levels by 24 h in transgenic rice (Bihani *et al.*, 2011). The transcript level of chickpea *CAP2* increased by dehydration, NaCl, ABA, and

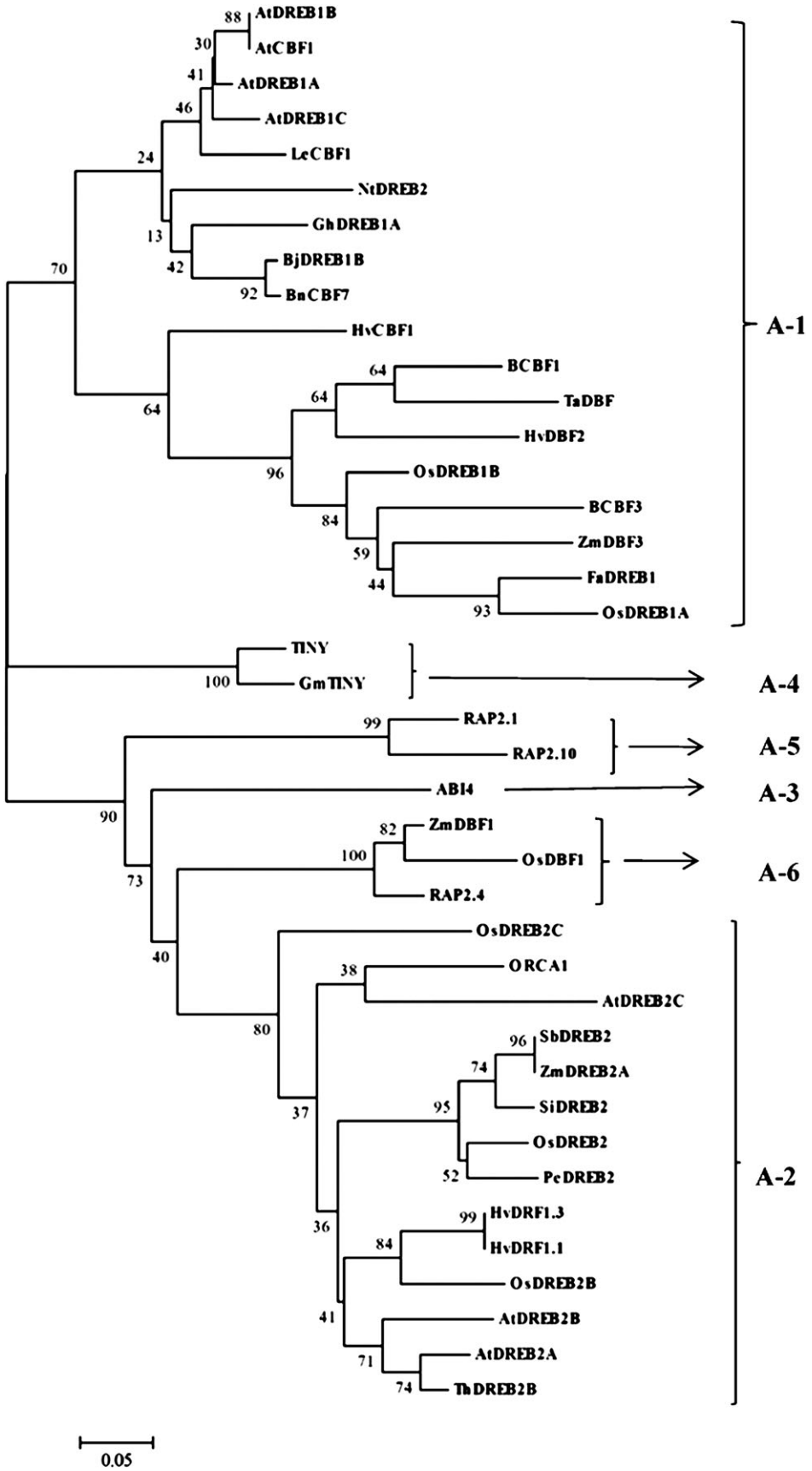

Fig. 3. Phylogenetic analysis of AP2/ERF domains of published DREB proteins in NCBI database. The phylogenetic tree was generated by MEGA 4.0 software. Branch lengths indicate distance. A-1 to A-6 indicate the groups proposed by Sakuma *et al.* (2002). The appended proteins are as follows: *Arabidopsis thaliana* AtDREB1 (BAA33434), AtDREB1B (BAA33435), AtDREB1C (BAA33436), RAP2.1

auxin treatments but not by treatments with low temperature, salicylic acid, and jasmonic acid (Shukla *et al.*, 2006). Chrysanthemum *DvDREB2A* was significantly affected by heat, low temperature, drought, ABA, and high salt treatments (Liu *et al.*, 2008). Expression of *Populus euphratica PeDREB2* was induced by cold, drought, and high salinity, but not by ABA (Chen *et al.*, 2009). The transcript expression of *SbDREB2A* from *Salicornia brachiata* was induced by NaCl, drought, and heat stress (Gupta *et al.*, 2010).

AtDREB2A accumulated in roots, stems, and leaves under normal growth conditions (Liu *et al.*, 1998). *DREB2C* expression was observed in mature embryo and the cotyledons of germinating seedlings (Lee *et al.*, 2010). Almoguera *et al.* (2009) reported that sunflower *HaDREB2* accumulates in all vegetative tissues. Chrysanthemum *DvDREB2A* was expressed in all organs under natural conditions with the highest transcript accumulation in flowers while less accumulation was detected in roots, stems, and young leaves (Liu *et al.*, 2008). *SiDREB2*, an A-2 type DREB family gene was expressed in leaves, roots, and young and mature spikelets of foxtail millet suggesting its role in developmental pathways also (Lata *et al.*, 2011*a*).

Stress tolerance through over-expressing DREBs

Functional analyses *in vivo* are important to understand the molecular mechanisms of stress tolerance in plants and also to provide tools that might improve crop productivity. One important way of achieving tolerance to multiple stress conditions is to over-express TFs that control multiple genes from various pathways. In fact, over-expression of several DREB TFs in transgenic plants using various promoters has resulted in plants more tolerant to drought, salt, heat, and freezing stresses (Table 3).

Transgenic *Arabidopsis* plants expressing *DREB1B*/*CBF1* or *DREB1A*/*CBF3* under the control of the cauliflower mosaic virus (CaMV) *35S* promoter showed strong tolerance to freezing, drought, and high salinity stresses suggesting that DREBs/CBFs target multiple genes (Jaglo-Ottosen *et al.*, 1998; Liu *et al.*, 1998; Kasuga *et al.*, 1999). *DREB1A*/*CBF3* over-expressing lines accumulated osmoprotectants, such as proline and various sugars, under non-stress conditions (Gilmour *et al.*, 2000). Transgenic *Arabidopsis* and rice plants over-expressing *OsDREB1A* were tolerant to low temperatures, high salinity, and drought (Dubouzet *et al.*, 2003; Ito *et al.*, 2006). However, over-expression of both *AtDREB1A* and *OsDREB1A* in *Arabidopsis* caused severe growth retardation under optimal growth conditions. The level of stress tolerance and growth retardation in the transgenic *Arabidopsis* over-expressing *OsDREB1A* was relatively lower compared with *AtDREB1A* which might be due to difference in the number of target stress genes induced (Agarwal *et al.*, 2006).

Over-expression of the *Arabidopsis DREB1*/*CBF* genes in transgenic *Brassica napus* or tobacco plants induced expression of downstream genes and increased the freezing tolerance of transgenic plants (Jaglo-Ottosen *et al.*, 2001; Kasuga *et al.*, 2004). *LeCBF1–3* (*DREB1*/*CBF* homologues) were reported to be present in a tandem array in the tomato genome (Zhang *et al.*, 2004). Out of these three genes only the *LeCBF1* gene was found to be cold-inducible and its constitutive over-expression in transgenic *Arabidopsis* plants induced expression of DREB1/CBF-targeted genes and increased freezing tolerance. On the other hand, over-expression of *CBF1*/*DREB1B* in tomato has been shown to increase the chilling and drought tolerance of transgenic tomato plants (Hsieh *et al.*, 2002*a*, *b*). A constitutive over-expression of either *LeCBF1* or *DREB1A* in transgenic tomato plants, however, did not result in increased freezing tolerance (Zhang *et al.*, 2004).

The over-expression of *DREB1A*/*CBF3* driven by the stress-inducible *rd29A* promoter in transgenic wheat improved drought stress tolerance (Pellegrineschi *et al.*, 2004). Similarly, the constitutive over-expression of *CBF3*/*DREB1A* using the *35S* promoter in transgenic rice resulted in increased stress tolerance to drought and high salinity without any growth inhibition or phenotypic aberrations (Oh *et al.*, 2005). The constitutive over-expression of *CBF4* with resultant induction of *cor* genes in *Arabidopsis* displayed higher tolerance for freezing and drought stress (Haake *et al.*, 2002). The *35S:TaDREB1* rice transgenic plants showed a dwarf phenotype, although the same was not observed in the corresponding *Arabidopsis* transgenic plants. Thus, it may be possible that a monocot gene transferred to dicots may not function as effectively as in the monocots (Shen *et al.*, 2003*a*).

Transgenic tobacco plants over-expressing *PpDBF1* showed higher tolerance to salt, drought, and cold stresses (Liu *et al.*, 2007). The over-expression of the *AhDREB1* gene led to the accumulation of its putative downstream target genes and also conferred a better survival rate to transgenic tobacco plants under salt stress compared with the wild-type plants (Shen *et al.*, 2003*b*). These results are also consistent

(NP564496), RAP2.10 (NP195408), RAP2.4 (NP177931), AtCBF1 (NP567721), AtDREB2A (AAU93685), AtDREB2B (BAA36706), AtDREB2C (Q8LFR2), ABI4 (AF085279), TINY (AAC29139); *Oryza sativa* OsDREB1A (AAN02486), OsDREB1B (AAX28958), OsDREB2 (AAN02487), OsDREB2B (Q5W6R4), OsDREB2C (Q84ZA1), OsDBF1 (AAP56252); *Zea mays* ZmDREB2A (ACG47772), ZmDBF1 (AAM80486), ZmDBF3 (NP 001105651); *Triticum aestivum* TaDBF (AAL37944); *Sorghum bicolor* SbDREB2 (ACA79910); *Setaria italica* SiDREB2 (HQ132744); *Hordeum vulgare* HvDBF2 (AAM13419), HvCBF1 (AAX23686), BCBF1 (AAK01088), BCBF3 (AAK01089), HvDRF1.1 (AAO38209), HvDRF1.3 (AAO38211); *Festuca arundinacea* FeDREB1 (CAG30550); *Phyllostachys edulis* PeDREB2 (ABY19376); *Lycopersicum esculentum* LeCBF1 (AAS77820); *Nicotiana tabacum* NtDREB2 (ACE73694); *Gossypium hirsutum* GhDREB1A (AAP83936); *Glycine max* GmTINY (ACP40513); *Catharanthus roseus* ORCA1 (CAB93939) *Brassica juncea* BjDREB1B (ABX00639), *Brassica napus* BnCBF7 (AAM18959); *Thellungiella halophila* ThDREB2B (ABV08790).

Table 3. Stress response of overexpressing DREBs in transgenic plants.

DREB gene	Transgenic plants	Stress response	References
AtDREB1A	*Arabidopsis*	Drought tolerance	Liu *et al.*, 1998
AtDREB1A	Tobacco	Freezing and drought tolerance	Kasuga *et al.*, 2004
AtDREB1A	Wheat	Drought tolerance	Pellegrineschi *et al.*, 2004
AtDREB1A	Rice	Drought and salinity tolerance	Oh *et al.*, 2005
AtDREB2A	*Arabidopsis*	None	Liu *et al.*, 1998
AtDREB1A	Potato	Salinity tolerance	Behnam *et al.*,2006
AtDREB1A	Peanut	Drought tolerance	Bhatnagar-Mathur *et al.*, 2006
AtDREB2A-CA	*Arabidopsis*	Drought tolerance	Sakuma *et al.*,2006a
AtCBF1	*Arabidopsis*	Salinity tolerance	Jaglo-Ottosen *et al.*, 1998
AtCBF1	Tomato	Drought and freezing tolerance	Hsieh *et al.*, 2002a
AtCBF1	*Brassica napus*	Freezing tolerance	Jaglo-Ottosen *et al.*, 2001
AtCBF1	Canola	Freezing tolerance	Jaglo *et al.*, 2001
AtCBF3	*Arabidopsis*	Freezing tolerance	Gilmour *et al.*, 2000
AtCBF4	*Arabidopsis*	Freezing tolerance	Haake *et al.*, 2002
AtDREB2C	*Arabidopsis*	Thermotolerance	Lim *et al.*, 2007
OsDREB1A	*Arabidopsis*	Drought, salinity and freezing tolerance	Dubouzet *et al.*, 2003
OsDREB2A	*Arabidopsis*	None	Dubouzet *et al.*,2003
OsDREB2B	*Arabidopsis*	Drought and thermotolerance	Matsukura *et al.*, 2010
OsDREB1F	Rice, *Arabidopsis*	Drought, salinity and freezing tolerance	Wang *et al.*, 2008
OsDREB1G	Rice	Drought tolerance	Chen *et al.*, 2008
ZmDREB2A	*Arabidopsis*	Drought and thermotolerance	Qin *et al.*, 2007
PgDREB2A	Tobacco	Hyperionic and hyperosmotic stresses	Agarwal *et al.*, 2010
BNCBF5	*Brassica napus*	Freezing tolerance	Savitch *et al.*, 2005
BNCBF17	*Brassica napus*	Freezing tolerance	Savitch *et al.*, 2005
AhDREB1	Wheat	Drought and salinity tolerance	Shen *et al.*, 2003b
GmDREB	*Arabidopsis*	Freezing tolerance	Shiqing *et al.*, 2005
LeCBF1	Tomato	None	Zhang *et al.*, 2004
LeCBF1	Tobacco	Freezing tolerance	Zhang *et al.*, 2004
GhDREB1	Tobacco	Drought, salinity and freezing tolerance	Shan *et al.*, 2007
PpDBF1	Tobacco	Drought and salinity tolerance	Liu *et al.*, 2007
CAP2	Tobacco	Salinity tolerance	Shukla *et al.*, 2006
PeDREB2	*Arabidopsis*	Salinity tolerance	Chen *et al.*, 2009
HvDREB1	*Arabidopsis*	Drought tolerance	Xu *et al.*, 2009

with the over-expression of *OsDREB1F* as tolerance to high salinity, drought, and low-temperature is enhanced both in rice and *Arabidopsis* transgenic plants (Wang *et al.*, 2008).

The over-expression of *DREB2A* does not result in any phenotypic changes in *Arabidopsis* transgenic plants, i.e. neither any retardation in growth nor any improved stress tolerance (Liu *et al.*, 1998). This suggested that the DREB2A protein requires post-translational modification such as phosphorylation for its activation (Liu *et al.*, 1998). Busk and Pagès (1998) also reported that phosphorylation may be necessary for the activation of proteins under drought-stress conditions, thus enhancing the DNA-binding activity of several transcription regulators. The central region of DREB2A contains a negative regulatory domain as revealed by its domain analysis using *Arabidopsis* protoplasts and that the internal deletion of amino acids 136 to 165 makes DREB2A constitutively active. Over-expression of this constitutively active form (*DREB2A-CA*) resulted in growth retardation in transgenic *Arabidopsis* plants, up-regulation of many stress-inducible downstream genes, and imparted significant tolerance to drought stress but only slight tolerance to freezing (Sakuma *et al.*, 2006*a*). The DREB2A-CA-GFP fusion protein showed a stable expression in the nucleus, however, the same was not the case with DREB2A-FL-GFP fusion protein, suggesting that the central region of DREB2A is required for the regulation of its stability (Nakashima *et al.*, 2009).

Although *DREB2A* and *DREB1A* were isolated together (Liu *et al.*, 1998), it was found that some of the downstream target genes of *DREB2A* were different from those of *DREB1A* (Nakashima *et al.*, 2009). The reason for this may be due to the fact that both DREB2A and DREB1A slightly differ in their DNA-binding specificities. While DREB2A preferentially binds ACCGAC motifs, DREB1A specifically has a high affinity to A/GCCGACNT sequences (Sakuma *et al.*, 2006*a*). Microarray analysis of transgenic *Arabidopsis* plants over-expressing *DREB2A-CA* revealed that its over-expression not only induced drought- and salt-responsive genes but also heat-shock (HS)-related genes. These transgenic plants also showed improved thermotolerance which was significantly decreased in *DREB2A* knockout plants (Sakuma *et al.*, 2006*b*). Moreover, it was

found that transient induction of the *DREB2A* occurred rapidly by HS stress, and that the sGFP-DREB2A (synthetic green fluorescent protein-DREB2A) protein accumulated in nuclei of HS-stressed cells. It was also observed that *DREB2A* up-regulated genes were down-regulated in *DREB2A* knock-out mutants under stress conditions (Yamaguchi-Shinozaki and Shinozaki, 2009). Over-expression of *DREB2C* was also found to induce the expression of many HS stress-inducible genes, ensuring thermotolerance of transgenic *Arabidopsis* (Lim *et al.*, 2007). Recently it has been shown that *HsfA3* (an HS TF) regulates expression of many heat-inducible genes downstream of the *DREB2A* stress-regulatory system and functions in acquiring thermo-tolerance under the control of the *DREB2A* transcriptional cascade (Schramm *et al.*, 2008; Yoshida *et al.*, 2008). Recent analyses of genetic responses involved in plant acclimation to high temperature have also indicated that *AtDREB2B*, together with *AtHSFA3*, are the only two TFs that were specifically induced in thermotolerant lines of *Arabidopsis*. Therefore, it appears that *AtDREB2A* and *AtDREB2B* could have some functional redundancy in thermo-tolerance, as mutants for either did not show a defect in heat acclimation (Schramm *et al.*, 2008); however, *dreb2a-1* mutant plants, showed reduced basal thermotolerance when directly treated at 46 °C for 45 min (Larkindale and Vierling, 2008). Taken together, it can be concluded that DREB2 regulons function in both osmotic and heat stress responses.

The over-expression of rice *OsDREB2A* does not result in any phenotypic changes in transgenic *Arabidopsis*, but over-expression of wheat *WDREB2* and maize *ZmDREB2A* caused such changes in transgenic *Arabidopsis* and tobacco (Dubouzet *et al.*, 2003; Kobayashi *et al.*, 2007; Qin *et al.*, 2007). Transgenic *Arabidopsis* plants over-expressing maize *ZmDREB2A* were dwarf, and exhibited improved drought and heat stress tolerance. Microarray analysis of these plants revealed that 28 of 44 up-regulated genes were common with transgenic *Arabidopsis* over-expressing *DREB2A-CA* (Qin *et al.*, 2007). Over-expression of *OsDREB2B* in transgenic *Arabidopsis* showed enhanced expression of *DREB2A* target genes and improved drought and heat-shock tolerance (Matsukura *et al.*, 2010). Transgenic tobacco plants over-expressing *PgDREB2A* exhibited enhanced tolerance to both hyperionic and hyperosmotic stresses. The transgenics also showed higher expression of downstream genes *NtERD10B*, *HSP70-3*, *Hsp18p*, *PLC3*, AP2 domain TF, *THT1*, *LTP1,NtHSF2*, and pathogen-regulated (*NtERF5*) factors with different stress treatments (Agarwal *et al.*, 2010).

Recently Bihani *et al.* (2011) reported that the constitutive expression of sorghum SbDREB2 driven by the CaMV *35S* promoter led to pleiotropic effects in rice and these transgenics did not set seed. However, the *rd29A:SbDREB2* rice plants set seed and the transgenics showed a significantly higher number of panicles compared with the wild-type rice plants. Phenological and agronomic traits were also not affected in *rd29A:SbDREB2* transgenic rice (Bihani *et al.*, 2011). Over-expression of sunflower *HaDREB2* in seeds did not enhance longevity in transgenic tobacco. The constitutively over-expressing *HaDREB2* could not increase thermotolerance in seedlings or lead to the accumulation of HSPs at normal growth temperatures. By contrast, when *HaDREB2* and *HaHSFA9* (sunflower Heat Shock Factor A9) were over-expressed together, positive effects on seed longevity were observed, apart from those observed with over-expression of *HaHSFA9* alone (Almoguera *et al.*, 2009). Over-expression of *CAP2* in tobacco improved growth and development, and tolerance to dehydration and salt stress of the transgenic plants (Shukla *et al.*, 2006). Surprisingly, expression of *CAP2* cDNA in yeast (*Saccharomyces cerevisae*) also enhanced heat tolerance, with increased expression of gene for heat shock factor 1 (*Hsf1*) and its target yeast heat shock protein 104 (*Hsp 104*) suggesting strong evolutionary conservation of the stress response mechanisms (Shukla *et al.*, 2009). In another interesting study it was reported that the recombinant *E. coli* cells expressing *SbDREB2A* exhibited better growth in basal LB medium as well as in medium supplemented with NaCl, PEG, and mannitol. The improved growth in recombinant *E. coli* cells could be due to the regulation of stress-regulated functional genes by this TF and certain interactions with transcriptional network in the bacterial cells, thus providing stress tolerance (Gupta *et al.*, 2010).

These observations suggest that the DREB proteins are important TFs in regulating abiotic stress-related genes and play a crucial role in imparting stress tolerance to plants. The *DREB1* and *DREB2* regulons can thus be used to improve the tolerance of various kinds of agriculturally important crop plants to drought, high-salinity, and freezing stresses by gene transfer.

Mechanisms of DREB gene regulation and stress tolerance in plants

In the earlier sections, it was discussed that DREB genes are involved in abiotic stress responses and impart stress endurance to plants, but the physiological and biochemical bases of such responses and their activation mechanisms are still a matter of investigation. In *Arabidopsis* a number of regulatory genes which are involved in CBF cold-responsive pathway have been isolated and analysed by combining genetic and molecular approaches (Fig. 2). The gene products which may function directly in transcription are: higher expression of osmotically responsive genes1 (*HOS1*), a negative regulator of *CBFs*; *FIERY2* (*FRY2*), a transcriptional repressor; and low expression of osmotically responsive genes2 (*LOS2*), a positive regulator in the pathway (Yang *et al.*, 2005). A putative *MYC ICE1* (Inducer of CBF expression 1)-like TF may play an important role in activating *CBF1*/*DREB1B*, *CBF2*/*DREB1C* and *CBF3*/*DREB1A* (Chinnusamy *et al.*, 2003). Its activation requires cold-induced phosphorylation, and may be regulated by *HOS1* which targets the ICE1 protein for ubiquitination and subsequent degradation (Dong *et al.*, 2006; Saibo *et al.*, 2009). Further, a SIZ1 (a SUMO E3 ligase)-dependent sumoylation can block ubiquitination of

ICE1 (Miura *et al.*, 2007), where sumoylation is a process that conjugates SUMO (small ubiquitin-related modifier) to a protein substrate. This alteration activates/stabilizes ICE1 protein, which facilitates its activity to control the expression of the CBF3/DREB1A gene (Saibo *et al.*, 2009). The *DREB1*/*CBF* genes were also found to be regulated by Ca^{2+}-related processes as mutations in *CAX1*, a Ca^{2+}/H^{+} transporter, and *CBL1*, a Ca^{2+}-sensor, affected the expression of *DREB1*/*CBF* genes (Albrecht *et al.*, 2003, Catala *et al.,* 2003). Using a reverse genetics approach, Novillo *et al.* (2004) showed that *CBF2*/*DREB1C* acts as a negative regulator of *CBF1*/*DREB1B* and *CBF3*/*DREB1A* gene expression as *cbf* mutants were tolerant to drought, salinity, and freezing stresses. Another negative regulator of *CBF*/*DREB* genes is *MYB15* which interacts with the promoter regions to repress their expression (Agarwal *et al.*, 2006).

The mechanisms by which *CBF*/*DREB1* gene express in response to low temperature has also been elaborated by Zhao *et al.* (2006) in *Brassica napus* providing a new perspective to the regulation mechanisms of the DRE-mediated signalling pathway in cold-stress responses. They have isolated two groups of DREB-like genes namely; Group I and Group II which were induced by low temperature, but Group I expressed earlier than that of Group II. The Group I DREBs could specifically bind to the DRE *cis*-acting element to activate the downstream genes in *Brassica*, while Group II DREBs were *trans*-inactive but retained the ability to bind DRE sequence. Interestingly, the DRE binding ability of the two groups was similar, as revealed by fluorescence quenching assays. The genes of both these groups worked in a competitive manner, where Group II could suppress the *trans*-activation activity of Group I DREB in a concentration-dependent manner, strongly suggesting that the Group I DREBs express at the early stage of cold stress to switch-on the DRE-mediated signalling pathway, whereas the Group II DREBs express at the later stage of cold stress to switch-off this pathway. Thus the low-temperature response through the CBF/DREB regulon is a tightly regulated mechanism to ward off any negative effects in plants. As a matter of fact, their uncontrolled expression in certain conditions may lead to dwarf phenotypes and reduced yields as well (Saibo *et al.*, 2009).

However, until now, the mechanism of activation of DREB2-type genes is not well-studied. It is assumed that not only transcriptional regulation but post-translational modification like phosphorylation may be necessary for the activation of this class of proteins under stress conditions (Fig. 2). It was also evident from the fact that the over-expression of *AtDREB2A* and *OsDREB2A* could not induce target stress-inducible genes (Liu *et al.*, 1998; Dubouzet *et al.*, 2003). The presence of a conserved serine/threonine-rich region adjacent to the AP2/ERF domain may act as a putative site for phosphorylation as mentioned earlier. A negative regulatory domain has been identified in the *AtDREB2A* under normal conditions, deletion of which makes the protein not only constitutively active under stress conditions but also capable of up-regulating a number of drought, salt or heat-stress-responsive downstream genes (Sakuma *et al.*, 2006*a*). The authors have suggested the presence of a PEST sequence (RSDASEVTSTSSQSEVCT-VETPGCV) in this negative regulatory domain that contained many phosphorylation target sites for protein kinases like PKC and CK2. Rogers *et al.* (1986) suggested that the PEST sequence generally acts as a signal peptide and its phosphorylation is necessary for protein degradation (Salmeron *et al.*, 2001). In contrast to *Arabidopsis* DREB2A protein, maize and pearl millet DREB2A do not contain any PEST sequence (Agarwal *et al.*, 2007; Qin *et al.*, 2007). An *in vitro* ubiquitination assay revealed that the DRIPs (DREB2A-interacting protein, C3HC4 RING domain-containing proteins namely DRIP1 and DRIP2) mediate the degradation of *DREB2A*. The DRIP proteins also function as E3 ubiquitin ligases and are thus capable of mediating DREB2A ubiquitination (Qin *et al.*, 2008). Over-expression of full-length DREB2A protein was more stable in *drip1* than in the wild-type background suggesting DRIP1 and DRIP2 as novel negative regulators in drought-responsive gene expression by targeting DREB2A to 26S proteome proteolysis (Nakashima and Yamaguchi-Shinozaki, 2010).

DREBs are one of the important genes for crop improvement either through engineering stress tolerance or through crop breeding strategies since it is the major TF that binds to the *cis*-acting elements of most of the osmotic stress-inducible genes responsible for providing osmotolerance to the plants under stress conditions (Hussain *et al.*, 2011). As revealed by microarray analyses, most of these target stress-inducible genes contained the conserved DRE or DRE-related core motifs in their promoter regions (Maruyama *et al.*, 2004). Both cDNA and Gene Chip microarrays have revealed more than 40 target genes of DREB1/CBF including TFs, phospholipase C, LEA proteins, KIN (cold-inducible) proteins, sugar transport proteins, desaturase, carbohydrate metabolism related proteins, osmoprotectant biosynthesis proteins, and protease inhibitors known to function in stress response and are thought to be responsible for the observed stress tolerance of the transgenic plants (Fowler and Thomashow, 2002; Maruyama *et al.*, 2004). A cDNA microarray analysis of transgenic *Arabidopsis* plants over-expressing *AtDREB1A* revealed that 12 genes expressed 2-fold higher than in the wild-type (Liu *et al.*, 1998), out of which six were stress-related genes namely, *rd29A, kin1, cor6.6*/*kin2, cor15a, cor47*/*rd17*, and *erd10*. While the other six genes showed similarity to acclimatization protein, *DC1.2*, enolase, cysteine proteinase inhibitor, and *erd4*. A similar study on transgenic *Arabidopsis* over-expressing *OsDREB1A* showed 2-fold higher expression of six stress-related genes namely, *cor15a*, *FLO5-21-F13*, *rd29A*, *rd17*, *AtGolS3*, and *FLO5-20-N18* (Kasuga *et al.*, 1999; Dubouzet *et al.*, 2003). Both the studies suggested that products of these genes may function in stress tolerance in plants. Several such studies have also revealed that the over-expression of *DREB* genes driven either by the CaMV *35S* or the *rd29A* promoter led to the accumulation of stress-inducible putative downstream genes such as LEA proteins and heat-shock-related

genes, thus providing enhanced stress tolerance to plants (Shen *et al.*, 2003*a*; Oh *et al.*, 2005; Bhatnagar-Mathur *et al.*, 2006; Sakuma *et al.*, 2006*a*, *b*; Schramm *et al.*, 2008). The accumulation and activation of these genes have been thought to adapt the plants to stress conditions. Furthermore, elevated contents of osmoprotectants such as free proline, and various soluble sugars like sucrose and raffinose, and metabolites like galactinol and *myo*-inositol in the over-expressor transgenic plants were also detected, suggesting that the enhanced stress tolerance of the transgenic lines were because of the prompt accumulation of these substances compared with wild type/control plants (Gilmour *et al.*, 2000; Shiqing *et al.*, 2005; Ito *et al.*, 2006).

Since several previous studies have revealed that over-expressing DREBs/CBFs enhances the expression of downstream target genes, especially those that encode for LEA proteins, including dehydrins and COR proteins, a comparison of stress tolerance initiated by DREBs and those initiated by LEA proteins would be particularly interesting at this stage. As the mechanism of stress tolerance initiated by DREBs has already been discussed in this section, the focus here is on the stress tolerance initiated by LEA protein genes. LEA genes are associated with water or cold stress in plants and are active in tissues containing high ABA levels (e.g. seeds) (Tunnacliffe and Wise, 2007). Transgenic rice plants expressing barley *HVA1*, a Group 3 LEA gene, showed enhanced tolerance to water and salt stress. The transgenics were able to maintain relatively high levels of RWC and suffered less EL from cells suggesting that HVA1 protein was able to protect cell membranes from damage during osmotic stress (Rohila *et al.*, 2002; Babu *et al.*, 2004). Barley *HVA1* was also able to confer better growth and higher WUE to transgenic wheat plants (Sivamani *et al.*, 2000). Similar protection against dehydration stress was conferred to transgenic rice expressing Group I and II LEA protein genes from wheat (Cheng *et al.*, 2002). It has also been found that DREB genes regulate the expression of specific LEA genes like *COR14B* in wheat (Morran *et al.*, 2011). This may be possible due to the fact DREBs/CBFs could possibly regulate the activity of other downstream TFs, which may then regulate the specific expression of *LEA* genes. Therefore, a change in the expression level of a single DREB/CBF gene could regulate expression levels of other TFs, which, in turn, could lead to the activation of several downstream target genes, thus conferring stress tolerance to plants.

Hence, it can be concluded that DREB genes are the central regulator of abiotic stress responses and tolerance in plants exposed to adverse conditions. Engineering DREBs would regulate the expression of many target osmotic stress-inducible genes as well as up-regulate a group of indigenous stress-responsive pathways that collectively would produce physiological and biochemical adaptations in plants, enabling them to adapt and acclimatize to osmotic stresses. Thus, as a whole, the tolerance level of plants would be enhanced if DREBs are genetically engineered compared with any other stress-inducible genes, making them the popular targets for genetic engineering and crop improvement. In our own laboratory it was recently found that *SiDREB2*, a DREB2-like gene from foxtail millet is a major QTL for dehydration stress tolerance as it contributes to more than one-quarter of the variation in LP which is an important biochemical marker for oxidative stress in plants (discussed in next section; C Lata, M Prasad, unpublished data).

Role of DREBs in crop improvement through marker-assisted selection

Marker-assisted selection (MAS) provides a strategy for accelerating the process of conventional crop breeding. The tagging of useful genes, such as those responsible for conferring resistance to plants, the synthesis of plant hormones, and abiotic stress, namely drought and salinity tolerance, is a major target for improving crop growth and productivity (Lopez *et al.*, 2003). Plant improvement, either by natural selection or plant breeding has always been based upon creating, evaluating, and selecting the appropriate combination of alleles (Svetlana *et al.*, 2007). Hence, with the use of molecular markers it is now easy to trace valuable alleles either in segregating or natural populations. The efficiency of MAS is influenced by several complex factors, such as recombination between the marker and the candidate gene, a low level of polymorphism between the parents with contrasting traits, and a lower resolution of QTL due to environmental interactions. Marker-assisted breeding is a rapid and accurate method for introgressing any gene from a donor line into the genomic background of a recipient line, providing a very effective tool for marker-assisted backcross (MABC) breeding (Collard and Mackill, 2008). A schematic representation of MABC scheme is shown in Fig. 4. Among the various DNA-based markers, SNPs can serve as a powerful tool for MAS and map-based cloning since they are highly stable markers and often contribute directly to a phenotype (Anderson and Lübberstedt, 2003; Kim *et al.*, 2005). Among the several SNP genotyping methods, allele-specific PCR largely fulfils the basic requirements for MAS because of its simplicity, user friendliness, cost effectiveness, and reproducibility. Introduction of additional mismatch bases has played an important role in enhancing the specificity of this technique for an accurate discrimination of different alleles in MAS (Drenkard *et al.*, 2000).

Some of the recent studies have shown the importance of DREB TFs in marker-aided breeding and crop-improvement strategies. Functional markers in common wheat (*Triticum aestivum*) were designed based on genome-specific primers for each of the orthologous *Dreb1* loci on chromosomes 3A, 3B, and 3D to represent locus-specific differences, and the *Dreb-B1* locus was mapped on the long arm of chromosome 3B (Wei *et al.*, 2009). This genetic mapping of *Dreb-B1* on chromosome 3B may be useful in wheat breeding programmes aimed at improving its drought tolerance. In another study, drought-tolerant alleles developed for *DREB2* in common wheat produced clear, strong, reproducible signals, which were easy to score in a segregating population (Bibi *et al.*, 2010). In our laboratory, a synonymous SNP at the 558th bp position

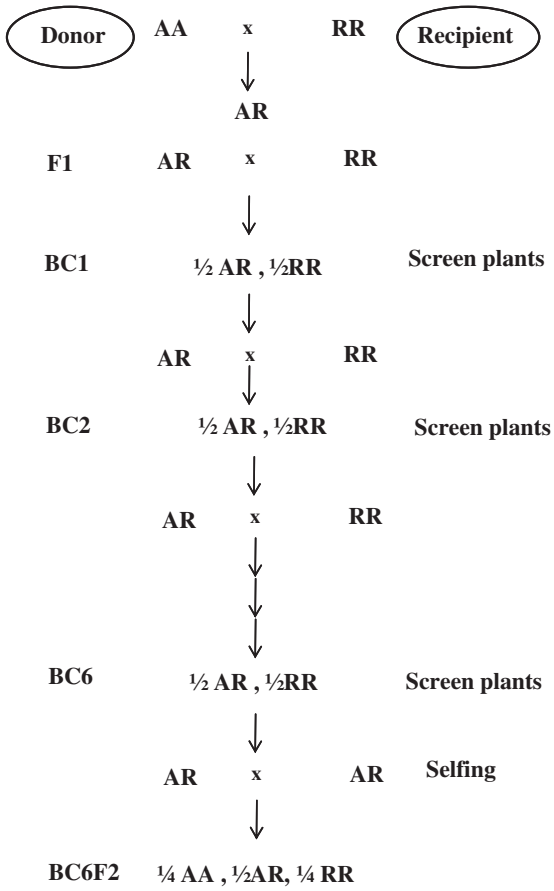

Fig. 4. A schematic representation of marker-assisted backcross (MABC) scheme.

(A/G transition) has been identified in the *SiDREB2* gene from dehydration-tolerant and -sensitive cultivars in foxtail millet (Lata *et al.*, 2011*a*). The dehydration-tolerant cultivars had an 'A''' while the sensitive cultivars had a 'G' allele. Based upon this sequence analysis, locus-specific (LSM) and allele-specific markers (ASM) were developed (Lata *et al.*, 2011*a*) and the association was studied further in an additional 61 and 101 dehydration-tolerant and -sensitive cultivars, respectively (C Lata, M Prasad, unpublished data). It is suggested that the ASM is tightly linked with LP, as the *SiDREB2*-associated trait contributed to 27.2% of the total variation in LP among the 170 cultivars (C Lata, M Prasad, unpublished data; and data not shown). The fact that the above ASM controlled more than one-quarter of the total variation in LP, it can be considered as a major QTL for dehydration tolerance in foxtail millet. Further, the ASM is part of the candidate gene, thus it eliminates the main disadvantage of MAS and, with the help of this marker, dehydration-tolerant cultivars can be selected at any stage. Furthermore, allele mining is a technique that exploits the DNA sequences of one genotype to isolate useful and valuable alleles from related genotypes. Such studies could also provide the foundation for plant breeding and translational genomic approaches (Latha *et al.*, 2004). Therefore, the *SiDREB2*-ASM would facilitate allele-mining of foxtail millet germplasm resources, thus leading to the identification, utilization, and introgression of newer alleles in crop improvement.

Conclusions

Understanding the molecular mechanisms of plant responses to abiotic stresses such as, drought, salinity, heat, and cold is very important as it helps in manipulating plants to improve stress tolerance and productivity. In response to these stresses, many genes are regulated mainly by TFs and their gene products function in providing stress tolerance to plants. One such class of the TFs is DREB/CBF that binds to DRE *cis*-acting elements. In this review, we have summarized that DREB genes are important plant TFs that regulate various stress-responsive gene expression. They play a key role in providing tolerance to multiple stresses, generally in an ABA-independent manner through DRE/CRT *cis*-elements and the AP2/ERF DNA binding domain. The DREBs can be genetically engineered to produce transgenics with higher tolerance to drought, salinity, heat, and cold using different promoters. Functional analysis of DREB TFs will provide more information on the complex regulatory networks involved in abiotic stress responses and the cross-talk between different signalling pathways during the adaptation of plants to various abiotic stresses. In addition, considering DREBs as candidate genes and developing proper functional markers, which could eventually be used for MAS and allele-mining in breeding programmes, will lead us to develop crop varieties superior in stress tolerance by genetic manipulation.

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